The in vitro reduction of sodium [36Cl]chlorate in bovine ruminal fluid

C. E. Oliver, M. L. Bauer, J. S. Caton, R. C. Anderson and D. J. Smith

J Anim Sci 2007.85:2059-2068.
doi: 10.2527/jas.2007-0056 originally published online May 15, 2007;

The in vitro reduction of sodium [^{36}Cl]chlorate in bovine ruminal fluid[1,2]

C. E. Oliver,* M. L. Bauer,* J. S. Caton,* R. C. Anderson,†[3] and D. J. Smith‡[4]

*Department of Animal and Range Sciences, North Dakota State University, Fargo 58105;
†Food and Feed Safety Research Unit, USDA-ARS, College Station, TX 77845; and
‡Biosciences Research Laboratory, USDA-ARS, Fargo, ND 58105

ABSTRACT: Sodium chlorate effectively reduces or eliminates gram-negative pathogenic bacteria in the gastrointestinal tracts of live cattle. Limitations to the in vivo efficacy of chlorate are its rapid absorption from the gastrointestinal tract and its presumed reduction to chloride within the gastrointestinal tract. We hypothesized that chlorate would be reduced via ruminal bacteria in a ruminal in vitro system and that the reduction of chlorate would be influenced by the dietary forage:concentrate ratio; thus, 4 ruminally cannulated steers were fed 20 or 80% concentrate diets in a crossover design. Ruminal fluid was collected in 2 periods and dispensed into in vitro tubes containing sodium [^{36}Cl]chlorate, which was sufficient for 100 or 300 mg/L final chlorate concentrations. The tubes were incubated for 0, 1, 4, 8, 16, or 24 h; autoclaved, control ruminal fluid, fortified with sodium [^{36}Cl]chlorate, was incubated for 24 h. Chlorate remaining in each sample was measured by liquid scintillation counting after [^{36}Cl]chloride was precipitated with silver nitrate. A preliminary study indicated that chlorite, a possible intermediate in the reduction of chlorate, had a half-life of approximately 4.5 min in freshly collected (live) ruminal fluid; chlorite was, therefore, not specifically measured in ruminal incubations. The chlorate dose did not affect in vitro DM digestion ($P \geq 0.11$), whereas in vitro DM digestibility was decreased ($P \leq 0.05$) by 80% forage content. By 24 h, 57.5 ± 2.6% of the chlorate remained in 100-mg/L incubations, whereas 78.2 ± 2.6% of the chlorate remained in the 300-mg/L incubations. When the data were expressed on a concentration basis (mg/L), diet had no effect ($P \geq 0.18$) on chlorate reduction; however, when chlorate reduction was expressed on a percentage basis, chlorate reduction tended to be greater ($P \geq 0.09$) at 8 and 16 h in the incubations containing the low-concentrate diet. Chlorate remaining in autoclaved controls at 24 h was intermediate ($P < 0.01$) between chlorate remaining in live ruminal fluid samples incubated for 0 or 24 h. Attempts to isolate chlorate-respiring bacteria from 2 sources of ruminal fluid were not successful. These data indicate that microbial-dependent or chemical-dependent, or both, reduction of chlorate occurs in bovine ruminal fluid and that dietary concentrate had a negligible effect on chlorate reduction.

Key words: bovine, chlorate, ruminal fluid

J. Anim. Sci. 2007. 85:2059–2068
doi:10.2527/jas.2007-0056

INTRODUCTION

Chlorate is a synthetic chloroxyanion that has potential in preharvest food safety applications (Callaway et al., 2003). Chlorate is hypothesized to be reduced to the cytotoxic chlorite ion by the enzyme respiratory nitrate reductase, which is found in *Enterobacteriaceae,* including *Escherichia coli* and *Salmonella* (Anderson et al., 2000). When administered in feed or drinking water to cattle, sheep, swine, or broilers, chlorate may cause a several-logarithm reduction in pathogen numbers or may eliminate experimentally introduced *E. coli* O157:H7 and *Salmonella* Typhimurium from the gas-

[1]We are grateful to Mike Giddings and Jason Holthusen for their technical assistance and to Jenn Leupp for helping with animal care. The assistance of Chelsea Pratt and Justin Terrell with microbiological work is also appreciated. This research was funded in part by beef and veal producers and importers through their $1-per-head checkoff and was produced for the Cattlemen's Beef Board and state beef councils by the National Cattlemen's Beef Association.

[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

[3]Holder of US patent 6,761,911 B2, "Use of chlorate ion or preparations thereof for reduction of food borne pathogens," assigned July 13, 2004.

[4]Corresponding author: david.j.smith@ars.usda.gov

Received January 22, 2007.
Accepted April 26, 2007.

trointestinal tract (Anderson et al., 2001a,b; Callaway et al., 2002, 2003; Jung et al., 2003).

Previous studies in cattle (Smith et al., 2005b; Oliver et al., 2007) have demonstrated that chlorate is rapidly and extensively removed, by absorption or reduction to chloride, from proximal portions of the gastrointestinal tract. This removal results in chlorate concentrations in the distal gastrointestinal tract and feces that are low relative to concentrations shown to be effective in vitro. In addition, a large portion of the chlorate dosed orally to cattle is reduced to chloride, but whether chlorate reduction occurs before absorption is unknown.

A likely site of chlorate reduction within the gastrointestinal tract is the rumen. Chlorate-reducing bacteria seem to be ubiquitous in many and diverse ecosystems, from pristine lakes to animal waste lagoons (Coates et al., 1999). The rumen, in addition to being a habitat for vast numbers and species of microorganisms, has a typical redox potential of –250 to –450 mV (Owens and Goetsch, 1988), suggesting that reduction of chlorate to chloride by chemical means is possible.

Our specific objectives were to determine whether chlorate reduction occurs within the rumen itself, to evaluate the effect of dietary forage concentration on in vitro chlorate reduction, and to isolate chlorate-reducing bacteria from ruminal fluid.

MATERIALS AND METHODS

Reagents and Chemicals

Radiolabeled sodium chlorate ($Na[^{36}Cl]O_3$), having a specific activity of 0.575 mCi/mmol, was purchased from Ricerca Biosciences (Concord, OH) and stored at 4°C until use. Unlabeled sodium chlorate [99.96% $NaClO_3$, 0.03% NaCl, and 0.01% H_2O] was acquired from EKA Chemicals (Columbus, MS) and Sigma (St. Louis, MO) and stored at room temperature. Other chemicals used in the study were sodium chloride (VWR, West Chester, PA), potassium phosphate dibasic anhydrous, ammonium phosphate monobasic, magnesium sulfate heptahydrate, ferrous sulfate heptahydrate, zinc sulfate heptahydrate, calcium chloride dihydrate, manganese (II) chloride tetrahydrate, boric acid, cobalt (II) chloride hexahydrate, sodium molybdate dihydrate, cupric sulfate pentahydrate, sodium selenite, nickel (II) chloride hexahydrate, sodium acetate anhydrous, silver nitrate, ammonium acetate (Sigma), trichloroacetic acid, sulfuric acid (99.999%), EDTA disodium salt dihydrate (Sigma), diphenylamine (99%, Sigma), sodium phosphate monobasic monohydrate (Acros Organics, Morris Plains, NJ), sodium phosphate dibasic, potassium chloride, magnesium sulfate, calcium chloride (Fisher Chemical, Fairlawn, NJ), sodium bicarbonate (Curtin Matheson Scientific, Baltimore, MD), NaOH (50% solution for ion chromatography, Fluka Chemical Corp., Milwaukee, WI), methanol (HPLC grade, EM Science, Gibbstown, NJ), bacto agar, resazurin (Difco, Becton Dickinson, Franklin Lakes, NJ), and Ultima Flo and Ultima Gold liquid scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA).

General Liquid Scintillation Counting Techniques

Background and limit of quantitation determinations and instrument calibration were described by Smith et al. (2005b). Samples were counted for 10 or 15 min on a Packard 2550 (Meriden, CT) or a Beckman LS1701 (Fullerton, CA) liquid scintillation counter. Quench was corrected using the H# (Beckman) or tSIE (Packard) options for each instrument.

Chlorate Recovery from Proprietary Formulation

Excess radiolabeled chlorate (114 dpm/μg) formulated for a previous study (Smith et al., 2005a) was recovered for use in this experiment. Briefly, the excess proprietary formulation was weighed and extracted into 75 mL of nanopure water (Milli-Q water purification system, Millipore Corp., Bedford, MA) over a 30-min period. The aqueous extract (286 μCi) was transferred to 2 pear-shaped flasks, and the water was completely evaporated using a rotary evaporator.

Purification of Recovered [^{36}Cl]Chlorate

A 2.5 × 63 cm glass column was packed with Sephadex G-10 (Pharmacia Inc., Piscataway, NJ) that was slurried in 0.1 *M* ammonium acetate. One-half of the dried residue was reconstituted in 5 mL of nanopure water and placed at the top of the Sephadex column. The column was eluted with 0.1 *M* ammonium acetate, pH 7.01, at a flow rate of 1.04 mL/min. Two hundred forty fractions of approximately 4 mL were collected at 3.8-min intervals. Aliquots (5 μL) of each fraction were diluted in 6 mL of Ultima Gold liquid scintillation fluid (PerkinElmer Life Sciences) and counted for 10 min each on a liquid scintillation counter. Radiochlorine in each vial was plotted by vial number to determine the elution of radioactivity, and the fractions (47 to 50) containing chlorate were combined in a 250-mL volumetric flask. The flask was diluted to the mark with nanopure water, mixed, and quintuplicate 100-μL aliquots were removed to determine recovery of radioactivity in the combined fractions. The chlorate solution was transferred to a 500-mL round-bottom flask, and the contents were dried using a rotary evaporator. The dried residue was reconstituted in nanopure water, transferred to a 25-mL volumetric flask, and the flask was brought to volume with nanopure water. Radiochemical purity was reassessed using ion chromatography, according to the method described in the section "Test article preparation and characterization" in Smith et al. (2005b); the radiochemical composition of the purified product was 99.8% [^{36}Cl]chlorate and 0.2% [^{36}Cl]chloride.

Chlorite Stability Experiment

Chlorate (ClO_3^-) has an oxidation state of +5. During its reduction to chloride (Cl^-; oxidation state of –1), 2

intermediates could be formed, chlorite (ClO_2^-; oxidation state of +3) and hypochlorite (ClO^-; oxidation state of +1). Hypochlorite is not stable enough to be detected as an intermediate form, because it is an extremely strong oxidizing agent; however, chlorite could be formed in ruminal fluid in measurable quantities. Because the analytical method we used to determine chlorate (see below) would also measure chlorite, it was important to know the stability of chlorite in freshly collected (live) ruminal fluid. Thus, [^{36}Cl]chlorite (99% radiochemical purity), synthesized as described by Hakk et al. (2007), was incubated in various media to ascertain its stability over time. Duplicate tubes containing [^{36}Cl]chlorite and either McDougall's buffer, autoclaved ruminal fluid (previously collected and frozen with no attempt made to maintain the autoclaved ruminal fluid in an anaerobic state), nanopure water, or live ruminal fluid were incubated in a 39°C water bath (Precision shallow form shaking bath, Jouan Inc., Winchester, VA) for 0, 20, 40, or 60 min; additional tubes were incubated for 5, 10, or 15 min for live ruminal fluid. Samples were removed from the water bath at the specified times and immediately filtered through 0.45-μm pore size, 17- or 25-mm diam., polytetrafluoroethylene syringe filters (Daigger, Vernon Hills, IL) into 2-mL screw-top, microcentrifuge tubes. Immediately after filtration, chlorite and chloride were separated and quantified by HPLC (600E pump/controller, Waters, Milford, MA). Aliquots of filtrate were injected (50 μL; Hamilton syringe, Reno, NV) onto AG-11 and AS-11HC guard and analytical columns (Dionex, Sunnyvale, CA). Chlorite and chloride were eluted using an isocratic (1 mL/min) mobile phase of 20 m*M* NaOH, and the radioactivity was detected (Packard model 500 TR flow scintillation analyzer with Ultima Flow AP scintillation fluid, Packard Bioscience, Boston, MA). Run times were 10 min with [^{36}Cl]chlorite (retention time 4 min) and [^{36}Cl]chloride peaks (retention time 5.7 min) integrated with Flo-One software (Packard Bioscience). Chlorite half-life was determined by fitting the chlorite stability data to a first order exponential decay regression equation ($Y = Y_0^{-kX}$; $R^2 = 0.98$) using GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

Animals and Study Design

Animal care, feeding, and ruminal fluid sampling protocols were approved by the North Dakota State University Animal Care and Use Committee.

Four ruminally cannulated yearling steers were fed 1 of 2 diets (20 or 80% concentrate) in a crossover design, with 12- to14-d diet adjustment periods and 14-d adaptation periods (total of 28 d between diet change and sample collection). For each feeding period, ruminal fluid was removed from the steers twice, on d 28 and 30 of the period, so that incubations within animal were replicated twice. Therefore, duplicate ruminal fluid samples were collected from 4 animals in each period.

Table 1. Composition of high- (80%) and low- (20%) concentrate diets (DM basis)

Item	High concentrate	Low concentrate
Diet ingredient	%	
Brome hay	20.00	80.00
Corn	67.14	12.23
Soybean meal	5.96	1.69
Concentrated separator by-product	5.00	5.00
Limestone	1.52	0.55
Dicalcium phosphate	—	0.15
Sodium chloride	0.25	0.25
Trace mineral premix[1]	0.07	0.07
Vitamin E premix[2]	0.02	0.02
Vitamin A and D premix[3]	0.02	0.02
Ionophore premix[4]	0.02	0.02
Diet composition		
CP,[5] %	12.9	12.1
ME,[6] Mcal/kg	2.94	2.54
Ca,[5] %	0.92	0.78
P,[5] %	0.49	0.58

[1]Trace mineral premix contained the following ingredients: 30 g/kg of copper sulfate; 45 g/kg of ferrous sulfate; 180 g/kg of zinc sulfate or zinc oxide; 128 g/kg of manganese sulfate or manganese oxide; 2.78 g/kg of ethylenediamine dihydroiodide or calcium iodate; and 0.563 g/kg of cobalt carbonate.
[2]Vitamin E premix contained 44,100 IU/kg of vitamin E.
[3]Vitamin A and D premix contained 48,488,000 IU/kg of vitamin A and 4,628,400 IU/kg of vitamin D_3.
[4]Diet formulated to provide 27.5 mg/kg of monensin.
[5]Values obtained from feed analysis.
[6]Value obtained by calculation from the NRC (1996) values.

Diet Composition

Low- (20%) and high- (80%) concentrate diets (Table 1) were formulated using the TAURUS ration program for beef cattle, version 1.01 (1990, The Regents of the University of California, Davis). A soybean meal-based supplement and premix was made for each diet. The supplements were mixed in a small Davis Precision horizontal batch mixer (H. C. Davis Sons Manufacturing Co. Inc., Bonner Springs, KS) and stored in open, plastic garbage pails. The diets were mixed in a larger Davis Precision horizontal batch mixer or a Roto-Mix IV feed mixer (model 84-8 STAT, Dodge City, KS). Hay and corn were weighed in the mixers with a Weigh-Tronix model 715 (Fairmont, MN) or a Digi-Star E2 2000 scale (Roto-Mix, Dodge City, KS) for the Davis and Roto-Mix mixers, respectively. Other ingredients were weighed on a scale cart from IQ Plus (model IQ+ 390-DC, Rice Lake Weighing Systems, Rice Lake, WI). Proximate analysis of each diet ingredient was performed by the North Dakota State University Animal and Range Sciences Nutrition Laboratory (AOAC, 1990). Diets were formulated to be 12.5 and 11.5% CP for the 80 and 20% concentrate diets, respectively; to have a 2:1 Ca:P ratio; and to have the same proportion of a trace mineral mix, all on a DM basis. Results of the proximate analysis are presented in Table 1.

Animal Handling and Feeding

Four cannulated steers (346 ± 35 kg of BW) were assigned randomly to the dietary treatment sequence. The steers were housed in individual 3.0 × 3.7 m pens with slatted concrete floors. Water and feed were available ad libitum.

Steers receiving the high-concentrate diet were adjusted to 80% concentrate over 14 d according to the following sequence: 50% concentrate for 2 d, 65% concentrate for 5 d, and 75% concentrate for 7 d, with a 14-d adaptation period before ruminal fluid collections on d 28 and 30. The following day, steers were crossed over on the diets. Steers crossing from the high- to the low-concentrate diet were fed a 50% concentrate diet for 2 d and then the 20% concentrate diet for the remainder of the period. Steers crossing from the low- to high-concentrate diets were adjusted up as previously described, but with 1 d at 50% and 4 d at 65% concentrate.

Preparation of In Vitro Tubes

Samples of hay, corn, and soybean meal were ground individually through a Wiley mill fitted with a 2-mm screen. The ground ingredients and concentrated sugar separator by-product were combined in the same proportions as the fed ration and stored in sealed containers at –20°C until use. A 0.25-g aliquot of the appropriate ground ration was weighed into 50-mL conical-bottomed polypropylene tubes (Sarstedt Inc., Newton, NC) with a 1.6-mm diam. hole in the screw-cap to allow gas to escape.

Ruminal Fluid Collection and Handling

Approximately 1.5 to 2 L of ruminal fluid was collected from each animal and placed into warmed 1-L thermoses on d 28 and 30 of each period, approximately 2 h after feeding. Ruminal fluid was collected by pushing the head of a suction strainer through the lip of the cannula plug into the rumen and under the ruminal fiber mat; fluid was drawn via vacuum using a hand pump.

Ruminal fluid was filtered through 4 layers of cheesecloth; 500 mL was diluted to 1 L with 500 mL of warmed, anaerobic McDougall's buffer (McDougall, 1948; Tilley and Terry, 1963); and the mixture was flushed with CO_2. Buffered ruminal fluid was maintained in a 39°C water bath under CO_2 until it was dispensed into tubes. An additional 200 mL of filtered, buffered ruminal fluid was autoclaved. To each incubation tube, 30 mL of live, buffered ruminal fluid was added. Autoclaved ruminal fluid controls and nanopure water controls were prepared separately.

Concentrations of sodium chlorate selected for inclusion in the in vitro incubations (100 and 300 mg/L) were based on chlorate doses from a previous study (Smith et al., 2005b), and the calculated ruminal chlorate concentrations were based on those doses. Additionally, the doses of chlorate shown to have in vitro efficacy against gram-negative pathogens (Anderson et al., 2000) were considered. Appropriate volumes of chlorate (27 or 80 μL, respectively) were added to each tube to yield 100 or 300 mg/L of chlorate. All tubes were prepared within 1.5 h of ruminal fluid collection, beginning with the longest incubations and working to the shortest. Each tube was flushed with a gentle stream of CO_2, and the tubes were then capped, mixed, and incubated at 39°C. The tubes were incubated for 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 16, or 24 h (1 tube for each steer, period, replicate, and dose for each time point) from the time of chlorate inoculation. The tubes were agitated by hand for about 15 s at approximately 1.5, 2.5, 4.5, 6.5, 8.5, 12.5, and 16.5 h. At the appropriate time points, the tubes were removed from the incubator, frozen in a dry ice-acetone bath, and transferred to a –20°C freezer until analysis.

In Vitro Digestion Assays

Ruminal fluid from each steer (1 replicate from each period) was used to conduct the in vitro digestibility analyses, according to the method of Tilley and Terry (1963; including in vitro blanks and controls), with the following modifications: no urea was added and the incubation lengths were 12 and 24 h instead of 48 h. Samples of the 2 test diets were used as substrates. Chlorate (100 or 300 mg/L) was added to half of the incubations to determine the effect of chlorate on in vitro digestibility.

Quantitation of Total Radioactivity and Parent Chlorate

For each ruminal fluid incubation, the total amount of radioactivity per unit volume was measured. Because ruminal fluid is a highly quenching matrix, samples were clarified before liquid scintillation counting. Thawed samples were centrifuged at 3,210 × *g* for 25 min in a Beckman GS-6R centrifuge. Duplicate 2-mL aliquots of each supernatant were loaded onto duplicate, preconditioned (10 mL of methanol followed by 10 mL of nanopure water) Bond Elut Mega ME-C-18 columns (2-g sorbant; Varian, Harbor City, CA) and eluted into 16 × 100-mm borosilicate glass tubes. Each solid-phase extraction column was rinsed with 2 mL of nanopure water, and the rinse was collected into its respective tube. Triplicate 250-μL aliquots of eluate were pipetted into 7-mL glass liquid scintillation vials, and 500 μL of nanopure water and 6 mL of Ultima Gold liquid scintillation fluid were added to each vial. The vials were dark-adapted for at least 1 h and counted for 10 min on a liquid scintillation counter.

Chlorate analysis was based on the widely divergent solubilities of the silver salts of chlorate and chloride in aqueous solution (CRC, 1982). Thus, using silver nitrate, the salt of [^{36}Cl]chloride, formed by the reduction of [^{36}Cl] chlorate, could be selectively precipitated, leaving silver chlorate salts in the sample supernatant.

Each sample set was prepared with duplicate blanks and fortified controls consisting of autoclaved ruminal fluid fortified with 25 μL of a standard containing 48.4% [^{36}Cl]chloride and 51.6% [^{36}Cl]chlorate. To eluates (~3.25 mL) prepared during the total radioactivity analysis, 750 μL of 0.15 *M* silver nitrate solution was added. The tubes were gently vortexed and then centrifuged at 2,000 × *g* for 20 min. The supernatant was drawn into a 5-mL syringe and filtered through a 0.45-μm pore size, 30-mm diam., polytetrafluoroethylene filter [Varian or Whatman (Clifton, NJ)] into a clean, prelabeled 16 × 100 mm borosilicate glass tube. Triplicate 900-μL aliquots of each sample were pipetted into 7-mL glass liquid scintillation vials. Six milliliters of Ultima Gold liquid scintillation fluid was added to each vial. The vials were dark-adapted for at least 1 h and then were counted for 10 min on a liquid scintillation counter. The background was subtracted from each sample, and chlorate as a percentage of total radioactivity was calculated.

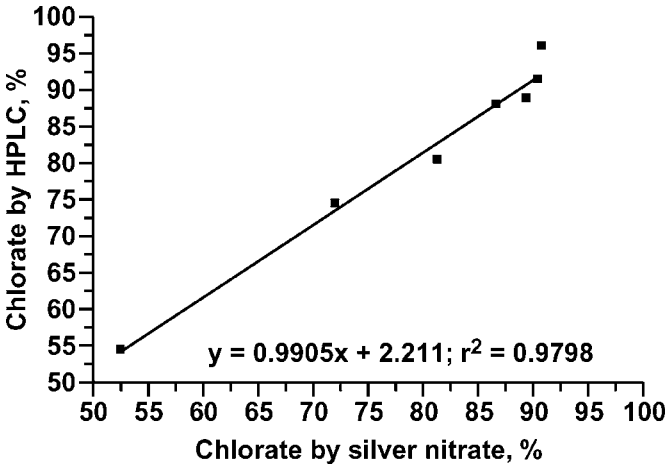

Figure 1. Validation of the silver nitrate chloride precipitation assay for chlorate. Samples were analyzed with both the silver nitrate-based assay and by ion chromatography, with the results of the assays regressed against each other.

Validation of Silver Nitrate Assay

To assure the accuracy of the silver nitrate assay, a representative set of samples was analyzed by ion chromatography with radiochemical detection and by the silver nitrate precipitation assay. A set of 4 in vitro incubation samples (representative of diet, chlorate dose, and period) and 2 autoclaved incubated samples were selected for inclusion in the analysis. In addition, a fortified control was prepared by spiking 5 mL of control ruminal fluid (autoclaved and pooled from the 4 steers) with 25 μL of a solution containing 48.4% [$^{36}Cl^-$] and 51.6% [$^{36}Cl]O_3^-$ (382 dpm/μL). Controls and validation samples were centrifuged at 3,210 × *g* for 25 min. Two-milliliter aliquots of each sample were loaded onto a preconditioned (10 mL of methanol followed by 10 mL of nanopure water) 12-mL Bond Elut Mega ME-C-18 column (2-g sorbant; Varian) and eluted into a prelabeled 20-mL glass liquid scintillation vial. Each column was rinsed with 2 mL of nanopure water, and the eluate was collected into its respective vial. Duplicate 100-μL aliquots of each eluate were pipetted into liquid scintillation vials. To each vial was added 1 mL of nanopure water and 15 mL of Ultima Gold liquid scintillation fluid. Vials were dark-adapted for at least 1 h and counted for 10 min in a liquid scintillation counter.

Duplicate 100-μL volumes of each sample eluate were also injected into the HPLC system described above. Radioactivity was eluted with 10 m*M* NaOH held isocratic for 10 min; from 10 to 32 min, the mobile phase composition was changed linearly to 55 m*M* NaOH. Fractions were collected in 20-mL glass liquid scintillation vials every 3 to 4 min without splitting the peaks. Each vial was counted as above. Recoveries of chlorate and chloride were calculated based on counts obtained from liquid scintillation analysis of an equal volume of unchromatographed eluate. Results of the HPLC analysis were regressed against the results of the silver nitrate assay for the same samples (Figure 1). The regression equation (y = 0.9905x + 2.2105) indicated a nearly 1-to-1 relationship (R^2 = 0.9798) between the 2 assay methods. The silver nitrate assay was also tested at chlorate:chloride ratios of 99:1 and 1:99. At these extremes, the assay overestimated chlorate levels but was accurate within <1% of the known values (data not shown).

Microbiological Isolation Procedure

Chlorate- and perchlorate-reducing bacteria have been previously isolated from numerous sources using a minimal medium in which the oxyanion of interest was the only electron acceptor; only those organisms that can use the oxyanion provided will grow in such a selective media (Rikken et al., 1996; Coates et al., 1999; Xu and Logan, 2003). To this end, a mineral salts medium (**MSM**) was made according to a modified recipe of Xu and Logan (2003) and Kengen et al. (1999). Stock solutions of the chemicals were prepared and combined to formulate a solution containing 1.18 g/L of K_2HPO_4, 0.85 g/L of $NaH_2PO_4 \cdot H_2O$, 0.5 g/L of $NH_4H_2PO_4$, 50 mg/L of $MgSO_4 \cdot 7H_2O$, 4 mg/L of $FeSO_4 \cdot 7H_2O$, 2 mg/L of $ZnSO_4 \cdot 7H_2O$, 1 mg/L of $CaCl_2 \cdot 2H_2O$, 1 mg/L of $MnCl_2 \cdot 4H_2O$, 0.6 mg/L of H_3BO_3, 0.4 mg/L of $CoCl_2 \cdot 6H_2O$, 0.33 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg/L of $CuSO_4 \cdot 5H_2O$, 0.15 mg/L of Na_2SeO_3, 0.1 mg/L of $NiCl_2 \cdot 6H_2O$, 3 mg/L of EDTA, 0.5 mg/L of resazurin, 1.39 g/L of Na acetate, and 0.64 g/L of $NaClO_3$. Appropriate amounts of each stock solution were combined in a beaker with added nanopure water. While stirring, the pH was adjusted to 7.0 with NaOH. The pH-adjusted solution was transferred to a volumetric flask, brought to volume with nanopure water, and mixed

by inverting the flask several times. The medium was transferred to a round-bottomed flask with a flask extension clamp on the flask neck. The flask was swirled over a Bunsen burner flame until the medium came to a rolling boil. The flask was removed from the flame, and N_2 gas was immediately bubbled through the medium. The flask was cooled in ice under N_2 gas for approximately 30 min. Ten milliliters of cooled medium was pipetted into 18 × 150 mm Hungate Type Anaerobic Culture Tube (Bellco Glass Inc., Vineland, NJ) under N_2 gas. The tubes were immediately sealed with a 20-mm septum stopper (Bellco Glass) with a tear-off aluminum seal (Bellco Glass) crimped over it. The tubes were autoclaved for 20 min (Amsco Eagle Series 3021, Steris Corp., Mentor, OH). Mineral salts agar plates were made by adding 2.2% Bacto agar to the MSM, autoclaving, tempering, and pouring into petri plates. When the agar was cooled, the plates were inverted and placed into an anaerobic chamber to reduce the agar.

A protocol for "Qualitative and Semi-Quantitative Spot Tests for Nitrate, Nitrite, Chlorate, Bromate, and Hypochlorite" was obtained from the Texas Veterinary Medical Diagnostic Laboratory in College Station and was developed into a quantitative colorimetric assay. Samples from the ruminal fluid-MSM incubations, chlorate standards [1, 2, 3, 4, 5, and 6 m*M* chlorate (107, 213, 320, 426, 533, and 640 mg/L of chlorate, respectively) prepared in ruminal fluid], and nanopure water blanks were pipetted (50 μL) into 1.9-mL microcentrifuge tubes. To each tube was added 250 μL of 10% trichloroacetic acid to precipitate protein. Tubes were centrifuged at 14,000 × *g* for 10 min at 4°C in a microcentrifuge (Hermle Z 180, Labnet International Inc., Edison, NJ). Duplicate or triplicate 50-μL aliquots of each standard, blank, and sample supernatant were pipetted into the wells of a labeled microtiter plate. A repeater pipet was used to dispense 150 μL of 0.5% diphenylamine [in 80% (vol/vol) sulfuric acid] into each well. The plate was shaken (VorTemp 56TM Incubator/Shaker, Labnet International Inc.) for 2 min at 100 rpm at room temperature. The plate was incubated at room temperature for ~15 min to let the color stabilize. Absorbance was measured at 700 nm in a microtiter plate reader (SpectraMax, Molecular Devices Corp., Sunnyvale, CA) using SoftMax Pro version 2.6 software (Molecular Devices Corp.). The assay was linear (R^2 = 0.99) from 1 to 6 m*M* (~100 to 650 mg/L) chlorate.

Ruminal fluid was collected from a pastured Jersey cow in College Station, Texas, and from a hay-fed beef steer in Fargo, North Dakota. Ruminal fluid from the beef steer was preserved in 20% glycerol and frozen before culture. In the laboratory, 2 mL of ruminal fluid from each animal was injected into a separate set of MSM tubes, 3 with chlorate and 3 without chlorate, using a 3-mL syringe and 16-gauge needle. The tubes were inverted several times to mix the contents. One-milliliter samples were collected with sterile 1-mL syringes and 18-gauge needles at 0, 24, 48, and 72 h and in a second experiment at 0, 48, 96, 144, and 192 h and frozen in labeled microcentrifuge tubes until analysis of chlorate by the diphenylamine colorimetric assay. The tubes were incubated at 39°C. At each of the collection times, the cultures were transferred to fresh media using anaerobic techniques. In the first experiment, at 72 h, 1 chlorate-containing culture was selected for plating. Serial dilutions were made in MSM media and plated (to concentrations of 10^{-1} to 10^{-9}) onto MSM agar in an anaerobic chamber. The plates were incubated anaerobically at 37°C.

Statistical Analyses

Results of chlorate degradation and in vitro DM digestion were analyzed by time with the GLM procedure (SAS Inst. Inc., Cary, NC). Replicate samples were averaged for statistical analysis. The model included animal, period, diet, dose, and the interaction of diet × dose. Least squares means were calculated for diet, dose, and the diet × dose interaction. In a separate analysis, the chlorate content of samples at 0 and 24 h were compared with the chlorate content of the 24-h autoclaved samples. The GLM procedure of SAS was used, and the model included animal, period, diet, dose, the interaction of diet × dose, and whether the sample was autoclaved. Least squares means were calculated for diet, dose, the diet × dose interaction, and the autoclaved vs. nonautoclaved comparison.

RESULTS

Chlorite Incubation Trial

When [^{36}Cl]chlorite was incubated in water, McDougall's buffer, or autoclaved ruminal fluid, no discernible pattern of reduction occurred within 1 h (data not shown), although chlorite in McDougall's buffer was lower ($P < 0.01$) across all time points including at time zero. Percentage chlorite remaining in replicates was averaged within media type across all time points. Thus, chlorite content of fortified water, McDougall's buffer, and autoclaved ruminal fluid was 98.2 ± 0.6%, 93.1 ± 2.2%, and 98.2 ± 0.8%, respectively, during a 1-h incubation at 39°C.

In contrast, chlorite incubated in live ruminal fluid was rapidly converted to chloride (Figure 2). Chlorite spiked into live ruminal fluid disappeared in less than 15 min. Average amounts of chlorite (percentage of initial) at 0, 5, 10, 15, and 20 min were 77.7 ± 20.4%, 43.4 ± 4.0%, 10.5 ± 7.8%, 0%, and 0%, respectively. The half-life of chlorite in the live ruminal fluid was about 4.5 min.

In Vitro DM Digestion

Concentrate and chlorate did not interact to affect ($P \geq 0.70$) in vitro DM digestibility. Chlorate did not affect ($P \geq 0.26$) in vitro DM digestibility at h 12 (52.0%, 51.3%, and 51.7% ± 0.89% for 0, 100, and 300 mg/L of

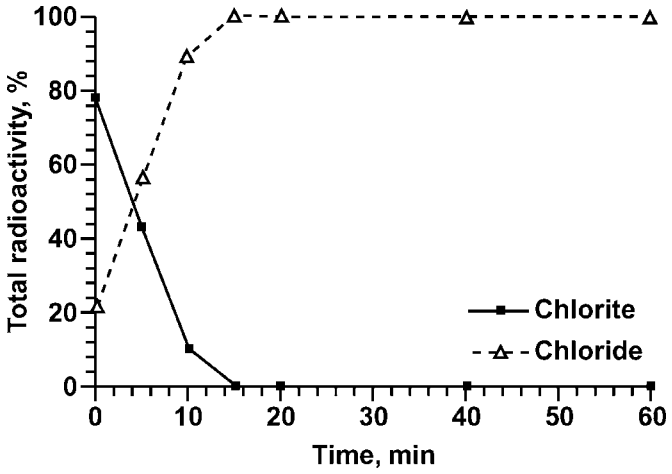

Figure 2. Reduction of chlorite and the appearance of chloride in live ruminal fluid over time. Results are the means of 2 replicates.

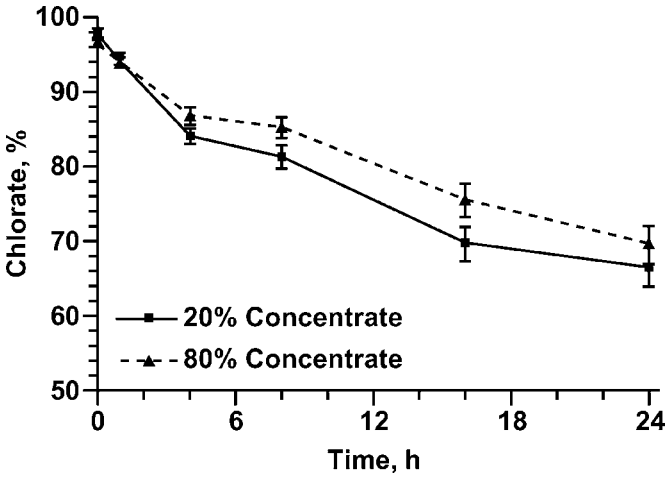

Figure 3. Effect of diet on ruminal in vitro chlorate degradation over time. Lines indicate the percentage of the initial chlorate remaining at the indicated times. Error bars are SEM.

chlorate, respectively) or 24 (68.2%, 66.8%, and 67.4% ± 0.55% digestible DM for 0, 100, and 300 mg/L of chlorate, respectively). Digestibility of the 20 and 80% concentrate diets differed ($P = 0.01$) at both 12 (43.0% vs. 60.4% ± 0.73% for 20 and 80% concentrate, respectively) and 24 h (56.7% vs. 78.2% ± 0.45% for 20 and 80% concentrate, respectively).

Chlorate Degradation

A diet × dose interaction did not occur ($P \geq 0.13$), so results of in vitro chlorate reduction in ruminal fluid incubations are presented as main effects. Diet type did not affect ($P \geq 0.18$) chlorate reduction when data were expressed and analyzed on a chlorate concentration (mg/L) basis (data not shown). When chlorate results were expressed and analyzed as a percentage of the total radioactivity, a tendency for a diet effect (Figure 3) was observed at h 8 ($P = 0.09$) and 16 ($P = 0.10$).

Because the high dose in this study was 3 times greater than the low dose, differences ($P = 0.01$) in chlorate concentrations between doses were expected. A more meaningful comparison between doses was obtained when the chlorate data were expressed on a percentage basis (Figure 4). When expressed on a percentage basis, there were differences ($P < 0.01$) at all but the initial time point ($P = 0.59$), indicating that a greater relative fraction of the low-chlorate dose was chemically reduced to chloride compared with the high-chlorate dose (Figure 4).

Chlorate reduction ($P = 0.01$) occurred in autoclaved ruminal fluid: by 24 h, approximately 17 and 6% of the beginning chlorate was reduced for the 100- and 300-mg/L doses, respectively. However, the extent of chlorate reduction in the autoclaved ruminal fluid was less ($P = 0.01$) than in the 24-h samples of live ruminal fluid.

Microbial Isolation Experiment

Bacterial growth was observed in MSM tubes, but there was less growth with successive transfers (data not shown). Colonies were observed on MSM agar plates only after 8 d of incubation. Colonies were small and colorless and did not grow when recultured in MSM. Tests for disappearance of chlorate in cultures showed that chlorate loss was practically nil (data not shown).

DISCUSSION

Chlorate reduction by nitrate reductase yields chlorite (Stewart, 1988). The assay used in this study for chlorate determination distinguishes chlorate from

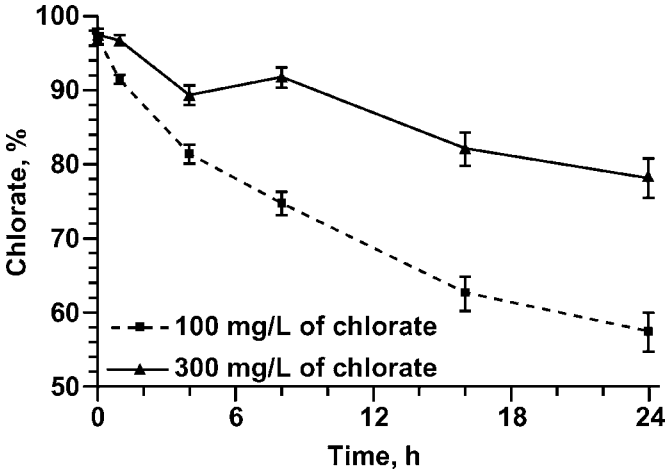

Figure 4. Effect of chlorate dose on ruminal in vitro chlorate degradation over time. Lines indicate the percentage of the initial chlorate remaining at the indicated time. Error bars are SEM.

chloride but not chlorite. It was therefore important to validate the accuracy of our chlorate assay by determining whether chlorite would likely be present in ruminal fluid as an intermediate in the reduction of chlorate. We are not aware of any other studies of chlorite stability in ruminal fluid. The results of our chlorite degradation study suggest that chlorite is relatively stable in the absence of live organisms for at least 60 min but that it is rapidly reduced to chloride by live ruminal bacteria (half-life ~4.5 min). Chlorite is also relatively unstable in rat and bovine urine and in rat and bovine serum (Hakk et al., 2007), presumably due to the presence of reductants within those matrices. It was somewhat surprising that chlorite was stable in autoclaved ruminal fluid in this study, but it is possible that our autoclaved ruminal fluid had no redox potential or a positive redox potential, because no effort was made to keep the ruminal fluid in an anaerobic state during its storage or in the autoclave. Due to the very rapid reduction of chlorite by ruminal microorganisms, we speculate that chlorite produced as a result of the ruminal reduction of chlorate would, at most, have only a transient existence (4.5 min half-life) and might be reduced so rapidly that it would not be detectable at all. Such observations are consistent with studies of perchlorate- or chlorate-reducing bacteria; in these bacterial populations, chlorite has never been detected as a metabolic intermediate due to the presence of chlorite dismutases (Malmqvist et al., 1991; Coates et al., 1999; Xu and Logan, 2003).

The fact that in vitro DM digestibilities were not affected by either dose of chlorate suggests that the majority of commensal ruminal microorganisms were not adversely affected by chlorate. Barry et al. (1978) found that in vitro ruminal cellulose digestion was decreased by 500 mg/L of potassium chlorate (1.4 to 5.0% compared with 9.2 to 11.1% disappearance of cotton thread with and without chlorate, respectively). Several other studies have shown that chlorate administration does not affect total culturable anaerobes present in the gastrointestinal tract, including the rumen (Anderson et al., 2001a,b; Callaway et al., 2002), but we cannot, with the available data from our study, speculate on how chlorate may have shifted the proportions of these ruminal commensal populations.

We are not aware of any other controlled studies that have measured ruminal chlorate metabolism. Harwood (1953) states that sodium chlorate is "deoxidized to sodium chloride" in the rumen but provides neither reference nor data to support his claim, nor does he speculate on whether this reduction is microbial or chemical. McCulloch and Murer (1939) were unable to detect $NaClO_3$ (sensitivity 0.01 mg/mL) in ruminal fluid of a sheep under postmortem examination that had received a 120-g dose 7 h before death, indicating that chlorate had been entirely absorbed, totally metabolized, passed downstream in the digestive tract, or a combination of the three. If the dose had been primarily metabolized, this report still does not provide any data to indicate whether the reduction was microbial or chemical in nature. Moore (1941), in discussing cases of chlorate poisoning in ruminants, proposes that different reducing bacterial types in the gut (and we suggest particularly in the rumen) may modify the effects of chlorate (we believe through differential metabolism of chlorate), thus explaining the variability in toxic levels of chlorate among individuals.

Our data indicate that reduction of chlorate does indeed occur in ruminal fluid regardless of whether the diet is primarily forage- or grain-based. Chlorate was also reduced in fresh autoclaved ruminal fluid but not to the same extent as in live ruminal fluid. These data suggest that the reduction of chlorate is at least partially due to a chemical reduction. The additional chlorate reduction observed in live ruminal fluid may be due to bacterial reduction or to a continual production of reductants by live bacteria, whereas the reducing equivalents in autoclaved ruminal fluid could have been limiting. Barry et al. (1978) reported that in vitro, daily addition of 500 mg/L of potassium chlorate increased the oxidation reduction potential of ruminal fluid day by day and that on a daily basis the oxidation reduction potential was greatest immediately after addition of chlorate. They also found that the oxidation reduction potential of in vivo ruminal fluid in response to daily (for 8 d) potassium chlorate dosing increased for the first 4 d and then returned to a predosing baseline (Barry et al., 1978). Short-term (3 d) larger doses increased oxidation state at first (d 1) but then caused a reducing state lower than baseline that persisted several days beyond discontinuation of chlorate dosing (Barry et al., 1978). Barry et al. (1978) concluded that long-term (8 d) administration of potassium chlorate is toxic to rumen microorganisms but that relatively large doses can be given for short periods (3 d). They speculated that a decreased fermentation rate in vitro may have caused the more positive reduction potential after several days of incubation (Barry et al., 1978). This speculation still leaves open both the possibility of an enzymatic reduction by bacteria (with fewer viable bacteria) or of a chemical reduction of chlorate (with a reduced capacity to generate reducing equivalents).

We were unsuccessful in insolating chlorate-respiring bacteria from the rumen. Lack of chlorate reduction in our MSM ruminal fluid cultures may indicate that a chlorate-reducing bacterium was not present, or it could mean that chlorate disappearance was not a sufficiently sensitive measure to detect low levels of potentially slow-growing bacteria. We are not aware of any other attempts to isolate a ruminal chlorate-respiring bacterium, but chlorate-reducing bacteria have been isolated from many and varied environments, including a swine waste lagoon, pristine aquatic sediment, Mississippi river sediment, pristine soil, gold mine drainage sediment, petroleum-contaminated soil, and a Florida swamp (Coates et al., 1999), and evidence has been discovered for chlorate-reducing bacteria in soil, ditch

water, river water, and sludge from waste water treatment plants (Åslander, 1928; van Ginkel et al., 1995).

Our samples were incubated up to 8 d. It is possible that longer incubation times were needed, because van Ginkel et al. (1995) found that in some cultures, up to 20 d of incubation was needed to induce bacterial chlorate reduction. Simon and Weber (2006) reported that greater than 1 m*M* perchlorate inhibited the mixed culture reduction of perchlorate for over 40 d. In contrast, at 0.01 and 1 m*M* perchlorate, the same culture reduced perchlorate completely after lag phases of 2 and 20 d, respectively. Our cultures were incubated with 6 m*M* chlorate, a concentration that may have been inhibitory to ruminal chlorate-respiring bacteria. A considerable amount of literature indicates that acetate is a critical component of the chlorate reduction reaction (Malmqvist et al., 1991; van Ginkel et al., 1995; Coates et al., 1999), but methanol, ethanol, and some AA (i.e., Gly, Val, Pro, Ala, Leu, and Asp) can also support chlorate reduction (Waki et al., 2004). The only electron donor in our media was acetate, and therefore, it may have been too selective to isolate any fastidious chlorate-respiring bacteria that may have been present.

Because the rumen is an anaerobic and reducing environment, we assumed that any potential chlorate-reducing bacteria would be anaerobic and would prefer a reducing environment. Although some perchlorate-reducing bacteria were isolated from anaerobic (Shrout et al., 2006; Simon and Weber, 2006) environments, many others have been isolated from aerobic environments (Coates et al., 1999), and some bacteria will reduce perchlorate even at redox potentials as high as +180 mV if sufficient electron donors are present (Shrout and Parkin, 2006). If a ruminal chlorate-reducing bacterium was microaerophilic, it might reside on the ruminal epithelium and would, therefore, have been difficult to isolate from ruminal fluid. Further research needs to be conducted to determine if there is a chlorate-respiring bacterium in the rumen and, if so, how it can be isolated.

A significant amount of chlorate is reduced to chloride in ruminal fluid. At least a portion of this reduction is by chemical means. The remaining reduction may be a result of chemical reduction from the continual production of reducing equivalents by ruminal bacteria or from a bacterial enzymatic reduction, although no such bacterium could be isolated from ruminal fluid. If chlorate were to be used as a preslaughter feed additive for decreasing the contamination of the carcasses of food animals with foodborne intestinal pathogens, ruminal chlorate reduction will decrease the amount of chlorate available for pathogen killing in the lower gastrointestinal tract. Therefore, development of a ruminally protected chlorate formulation for preslaughter food safety applications would be worthwhile.

LITERATURE CITED

Anderson, R. C., S. A. Buckley, T. R. Callaway, K. J. Genovese, L. F. Kubena, R. B. Harvey, and D. J. Nisbet. 2001a. Effect of sodium chlorate on *Salmonella* Typhimurium concentrations in the weaned pig gut. J. Food Prot. 64:255–258.

Anderson, R. C., S. A. Buckley, L. F. Kubena, L. H. Stanker, R. B. Harvey, and D. J. Nisbet. 2000. Bactericidal effect of sodium chlorate on *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 in rumen contents in vitro. J. Food Prot. 63:1038–1042.

Anderson, R. C., T. R. Callaway, S. A. Buckley, T. J. Anderson, K. J. Genovese, C. L. Sheffield, and D. J. Nisbet. 2001b. Effect of oral sodium chlorate administration on *Escherichia coli* O157:H7 in the gut of experimentally infected pigs. Int. J. Food Microbiol. 71:125–130.

AOAC. 1990. Official Methods of Analysis. 15th ed. Assoc. Off. Anal. Chem., Arlington, VA.

Åslander, A. 1928. Experiments on the eradication of Canada thistle, *Cirsium arvense,* with chlorate and other herbicides. J. Agric. Res. 36:915–934.

Barry, T. N., F. J. Harte, B. N. Perry, and D. G. Armstrong. 1978. Some effects of potassium chlorate administration on in vitro and in vivo rumen fermentation. J. Agric. Sci. Camb. 90:345–353.

Callaway, T. R., R. C. Anderson, K. J. Genovese, T. L. Poole, T. J. Anderson, J. A. Byrd, L. F. Kubena, and D. J. Nisbet. 2002. Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle. J. Anim. Sci. 80:1683–1689.

Callaway, T. R., T. S. Edrington, R. C. Anderson, K. J. Genovese, T. L. Poole, R. O. Elder, J. A. Byrd, K. M. Bischoff, and D. J. Nisbet. 2003. *Escherichia coli* O157:H7 populations in sheep can be reduced by chlorate supplementation. J. Food Prot. 66:194–199.

Coates, J. D., U. Michaelidou, R. A. Bruce, S. M. O'Conner, J. N. Crespi, and L. A. Achenbach. 1999. Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl. Environ. Microbiol. 65:5234–5241.

CRC. 1982. CRC Handbook of Chemistry and Physics. 63rd ed. R. C. Weast and M. J. Astle, ed. CRC Press, Boca Raton, FL.

Hakk, H., D. J. Smith, and N. W. Shappell. 2007. Tissue residues, metabolism, and excretion of $Na[^{36}Cl]O_3$ in rats. J. Agric. Food Chem. 55:2034–2042.

Harwood, P. M. A. 1953. Poisoning in dairy cattle by chemicals in common use. Vet. Rec. 19:291–292.

Jung, Y. S., R. C. Anderson, J. A. Byrd, T. S. Edrington, R. W. Moore, T. R. Callaway, J. McReynolds, and D. J. Nisbet. 2003. Reduction of *Salmonella* Typhimurium in experimentally challenged broilers by nitrate adaptation and chlorate supplementation in drinking water. J. Food Prot. 66:660–663.

Kengen, S. W. M., G. B. Rikken, W. R. Hagen, C. G. van Ginkel, and A. J. M. Stams. 1999. Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1. J. Bacteriol. 181:6706–6711.

Malmqvist, Å., T. Welander, and L. Gunnarsson. 1991. Anaerobic growth of microorganisms with chlorate as an electron acceptor. Appl. Environ. Microbiol. 57:2229–2232.

McCulloch, E. C., and H. K. Murer. 1939. Sodium chlorate poisoning. J. Am. Vet. Med. Assoc. 95:675–682.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43:99–109.

Moore, G. R. 1941. Sodium chlorate poisoning in cattle. J. Am. Vet. Med. Assoc. 99:50–52.

NRC. 1996. Pages 133–148 in Nutrient Requirements of Beef Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Oliver, C. E., A. L. Craigmill, J. S. Caton, R. C. Anderson, and D. J. Smith. 2007. Pharmacokinetics of ruminally-dosed sodium chlorate in beef cattle. J. Vet. Pharmacol. Ther. doi:10.1111/j.1365–2885.2007.00870.x.

Owens, F. N., and A. L. Goetsch. 1988. Ruminal fermentation. Pages 145–171 in The Ruminant Animal: Digestive Physiology and Nutrition. D. C. Church, ed. Prentice Hall, Englewood Cliffs, NJ.

Rikken, G. B., A. G. M. Kroon, and C. G. van Ginkel. 1996. Transformation of (per)chlorate into chloride by a newly isolated bacterium: Reduction and dismutation. Appl. Microbiol. Biotechnol. 45:420–426.

Shrout, J. D., and G. F. Parkin. 2006. Influence of electron donor, oxygen, and redox potential on bacterial perchlorate degradation. Water Res. 40:1191–1199.

Shrout, J. D., G. C. Struckhoff, G. F. Parkin, and J. L. Schnoor. 2006. Stimulation and molecular characterization of bacterial perchlorate degradation by plant-produced electron donors. Environ. Sci. Technol. 40:310–317.

Simon, R., and E. J. Weber. 2006. Reduction of perchlorate in river sediment. Environ. Toxicol. Chem. 25:899–903.

Smith, D. J., R. C. Anderson, D. A. Ellig, and G. L. Larsen. 2005a. Tissue distribution, elimination, and metabolism of dietary sodium [^{36}Cl]chlorate in beef cattle. J. Agric. Food Chem. 53:4272–4280.

Smith, D. J., C. E. Oliver, J. S. Caton, and R. C. Anderson. 2005b. Effect of sodium [^{36}Cl]chlorate dose on total radioactive residues and residues of parent chlorate in beef cattle. J. Agric. Food Chem. 53:7352–7360.

Stewart, V. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. 52:190–232.

Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestibility of forage crops. J. Br. Grassl. Soc. 18:104–111.

van Ginkel, C. G., C. M. Plugge, and C. A. Stroo. 1995. Reduction of chlorate with various energy substrates and inocula under anaerobic conditions. Chemosphere 31:4057–4066.

Waki, M., K. Suzuki, T. Osada, Y. Tanaka, M. Ike, and M. Fujita. 2004. Microbiological activities contributing to nitrogen removal with methane: Effects of methyl fluoride and tungstate. Bioresour. Technol. 94:339–343.

Xu, J., and B. E. Logan. 2003. Measurement of chlorite dismutase activities in perchorate-respiring bacteria. J. Microbiol. Methods 54:239–247.